ENCAPSULATION OF HEMOGLOBIN IN NON-PHOSPHOLIPID VESICLES

Vandegriff, K.D.[1,2], Wallach, D.F.H.[3], and Winslow, R.M.[1]
[1]Blood Cells, Inc., La Jolla, CA and [3]Microvesicular Systems, Nashua, NH
[2]Currently, the Division of Blood Research, Letterman Army Institute of Research, Presidio of San Francisco, CA 94129

ABSTRACT

The efficiency of encapsulating hemoglobin in non-phospholipid liposomes by rapidly mixing hemoglobin with lipids heated above their solid-liquid phase transition temperature was examined. Human hemoglobin was mixed at 55-60°C with a lipid solution containing polyoxyethylene-2 cetyl ether and cholesterol (molar ratio, 3:1) at 60-65°C. Repeated mixing was carried out through a high-shear orifice, followed by rapid cooling and additional mixing. Lipid vesicles were heterogeneous in size, with diameters from ~300 nm to 10 μm. The non-encapsulated aqueous phase was removed by centrifugation, and total hemoglobin was determined spectrophotometrically. Encapsulation efficiency was calculated as the percentage of hemoglobin associated with the liposome phase (*i.e.*, encapsulated) as a function of hemoglobin concentration and the aqueous:lipid hydration ratio. Hemoglobin concentrations were varied from 1 to 10 mM (in heme). Aqueous:lipid ratios of 8:1 and 4:1 were tested. Percent encapsulation varied from 13-30%, with the greatest efficiency, *i.e.*, 30%, at a 4:1 hydration ratio of hemoglobin:lipid at 5.6 mM hemoglobin.

INTRODUCTION

Red blood cells maintain sufficient oxygen-carrying capacity *in vivo* because of their high concentrations of intracellular hemoglobin. One approach in the design of red blood cell substitutes is to achieve efficient encapsulation ot high concentrations of hemoglobin in artificial membrane systems, or liposomes.

Biological membranes, including red blood cell membranes, are primarily phospholipid bilayers, combined with other membrane components. One critical component, the sterol cholesterol, increases membrane stability and fluidity. Thus, phospholipids and cholesterol are now the principal lipids used in liposome production. A common method for manufacturing liposome-encapsulated hemoglobin requires dissolving lipids, which are water insoluble, in an organic solvent, evaporating the solvent to leave a thin film of lipid, and then hydrating the dried lipids in a hemoglobin solution. The resulting multilamellar liposome suspension can be extruded through porous membrane filters to produce homogeneous unilamellar bilayer vesicles [1].

Large-scale processing and sterilization techniques in the manufacture of liposome-encapsulated hemoglobin have been problematic. Furthermore, pure phospholipids are expensive, and the presence of free phospholipids has been associated with toxicities of cell-free hemoglobin solutions [2]. A simpler approach to hemoglobin encapsulation, with a choice between either phospholipid or non-phospholipid components would be advantageous.

In this paper, we report the efficiency of hemoglobin encapsulation by a technique based on dissolution of lipids through temperature phase transition rather than in organic solvent. Both phospholipids and double or single-tailed non-phospholipid amphiphiles can be used to produce lipid microspheres called Novasomes™ [3]. Here, we discuss hemoglobin encapsulation with a lipid mixture containing a single-tailed non-phospholipid amphiphile, polyoxyethylene-2 cetyl ether, and cholesterol.

MATERIALS AND METHODS

Encapsulation procedure:

Solid polyoxyethylene-2 cetyl ether (POE-2) (ICI Specialty Chemicals) and cholesterol (R.I.T.A., Corp.), at a 3:1 molar ratio, were melted rapidly by heating to ~100°C and then allowed to cool to 60-65°C. Stroma-free human hemoglobin (provided by the Center for Blood Research, Boston, MA) was heated separately and rapidly in a water bath to 50-55°C. The lipid and hemoglobin solutions were then loaded into separate pre-heated syringes to the appropriate volumes for the hydration ratio (*e.g.*, for an 8:1 hydration ratio, 8 ml hemoglobin and 1 ml lipid). The temperature differential between lipid and aqueous phases for mixing was ≤10°C. The two liquid phases were mixed by pushing the lipid solution into the aqueous solution through the high-shear orifice of a 3-way metal stopcock. Mixing was continued for 20 strokes back and forth between the two syringes, followed by rapid cooling under cold water with continued mixing for 16-20 more strokes.

Washing procedure:

Unentrapped hemoglobin was separated from the Novasome layer by centrifugation at 3500 RPM for 10 min in 5% dextran-phosphate buffered saline at pH 7.4. The 5% dextran (87 kDa) imparts slightly greater density to the aqueous phase, giving a distinct separation between aqueous and Novasome layers, with the Novasome layer rising to the top. Wasning was repeated until hemoglobin was no longer detected in the wash solutions.

Determination of percent encapsulation:

Percent encapsulation was calculated from measurements of the total starting hemoglobin (in moles) minus the moles of hemoglobin washed free of the Novasome layer, after repeated washing, divided by the total. Hemoglobin concentration in each wash fraction was measured by absorbance at 523 nm (ε_{523} = 7.12 $mM^{-1}cm^{-1}$ [4]). A precise measurement of the volume of each wash fraction was determined by weight and density of the aqueous phase.

Denatured hemoglobin was observed as a gray particulate that precipitated during centrifugation. The amount of precipitated protein was unaccounted for and provides a source of error in the calculations.

Visual examinations:

Preparations were examined by light microscopy (400x) for analysis of homogeneity, vesicle aggregation, size and shape. Vesicle sizes were estimated by subjective comparison with calibrated polystyrene microspheres.

RESULTS

Percent encapsulations are given in **Table I**. Encapsulation efficiency decreased from 21 to 14% by increasing hemoglobin concentration from 1 to 10 mM. Decreasing the hydration ratio increased encapsulation of 10 mM hemoglobin to 22%. The highest percentage of encapsulation (30%) was observed with a 4:1 hydration ratio and 5.6 mM hemoglobin.

Table I

Percent encapsulation of Hemoglobin

Liposome formulation (Molar ratio)	Hydration ratio (aqueous:lipid)	[Hb] (mM)	% (final after washing)
POE-2/cholesterol (3:1)	8:1	1	21.0 ± 3.0[a]
POE-2/cholesterol (3:1)	8:1	10	13.5 ± 0.5[a]
POE-2/cholesterol (3:1)	4:1	10	22
POE-2/cholesterol (3:1)	4:1	5.6	30

[a]Average of 2 trials ± the range.

Light microscopy revealed heterogeneous vesicles, ranging in size from ~0.3-10 μm in diameter. Because the vesicles are osmotically active, their size and shape depend on the ionic composition of the suspension medium.

DISCUSSION

Encapsulation efficiency was calculated from the total initial hemoglobin minus free hemoglobin in the aqueous phase separated from the Novasome layer by centrifugation divided by the initial total hemoglobin. We have not shown that the hemoglobin associated with the Novasome layer is entirely intravesicular; some of the protein may be bound to the outside layer of the lipid membrane. To try to test for this, we prepared a control for each Novasome formulation: Novasomes made using buffer without hemoglobin were mixed afterwards with the hemoglobin solution in a test tube (*i.e.*, low shear) and subjected to the same washing procedure. Within the errors of our calculations, no hemoglobin was found to be associated with these Novasome preparations under any conditions after washing. This may not be conclusive, however, if high-shear mixing is a requisite for hemoglobin association with the outer surface of the lipid bilayer.

Percent encapsulation decreased as hemoglobin concentration was increased and increased as the hydration ratio was decreased. A reduction in the hydration ratio from 8:1 to 4:1 increases the amount of lipid relative to the aqueous phase and thus provides a higher degree of encapsulation. At high concentration, *i.e.*, 10 mM hemoglobin, the lipid/aqueous solution was viscous and manual mixing could have been inadequate. An automated process that supplies consistent hydrodynamic mixing forces might improve encapsulation efficiencies at high hemoglobin concentrations and/or decrease heterogeneity in vesicle size.

The hemoglobin denaturation observed in these experiments probably resulted from heating and/or mechanical shear. The detrimental effect of

heating on hemoglobin solutions may be circumvented in the future by using a modified hemoglobin, such as a cross-linked hemoglobin, with improved heat stability [5].

The method described here is fast, simple and inexpensive: (1) it requires unsophisticated techniques of heating and mixing, (2) non-phospholipids can be used, (3) organic solvents are not required, (4) encapsulation efficiencies are reproducible but may vary as a function of hemoglobin concentration or hydration ratio, and (5) viral inactivation potentially may be achieved during the phase-transition heating step.

The experiments described here were conducted on a small scale, *i.e.*, 10 ml, but large-scale production, using the same procedure, should be feasible with appropriate re-engineering of the process.

Author's (KDV) note: *The opinions or assertions contained herein are the private* views of the authors and are not to be construed as official or reflecting the views of the U.S. Department of the Army or the Department of Defense.

REFERENCES

[1] M.C. Farmer and B.P. Gaber, *Meth. Enzymol.* **149** 184-200 (1987).

[2] M. Feola, J. Simoni, P.C. Canizaro, R. Tran, G. Raschbaum, and F.J. Behal, *Surg. Gynecol. Obstet.* **166**, 211-222 (1988).

[3] D.F.H. Wallach and J.R. Philipott, Liposome Technology 2nd Edition (Ed., G. Gregoriadis) Boca Raton: CRC Press, pgs. 141-156 (1993).

[4] S.M. Snell and M.A. Marini, *Biochem. Biophys. Meth.* **17**, 25-34 (1988).

[5] T.N. Estep, M.K. Bechtel, T.J. Miller, and A. Bagdasarian, Blood Substitutes (Eds., T.M.S. Chang and R.P. Geyer) New York: Marcel Dekker, pgs. 129-134 (1989).